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IL-6 Modulates Alpha-Smooth Muscle Actin Expression in Dermal Fibroblasts from IL-6-Deficient Mice

Randle M. Gallucci[1], Eric G. Lee[1] and James J. Tomasek[2]

IL-6 deficient (IL-6KO) mice display significantly delayed cutaneous wound closure. Myofibroblasts are the primary mediators of wound closure, and alpha-smooth muscle actin (α-SMA) is a marker of fibroblast differentiation to the myofibroblast phenotype. Wounds from IL-6KO, and wild-type mice were collected up to 6 days following wounding. Expression of α-SMA mRNA was found to be increased in wounds of IL-6KO mice up to 48 hours post wounding, but decreased below wild-type levels by 72 hours. Recombinant IL-6 treatment of IL-6KO dermal fibroblasts showed an induction of α-SMA mRNA and protein peaking at 1 ng/ml cytokine, but declining at higher concentrations. Actinomycin-D treatment of fibroblast cultures indicated that recombinant mouse IL-6 (rmIL-6) induction of α-SMA mRNA appeared to be primarily transcriptionally regulated, and extracellular signal-regulated kinase 1/2 kinase, but not signal transducers and activators of transcription 3 was readily phosphorylated in rmIL-6 treated IL-6KO fibroblasts. A dose–response increase in the mRNA expression of the IL-6R signaling inhibitor protein suppressors of cytokine signaling (SOCS) 3 was also noted in rmIL-6-treated IL-6KO fibroblasts. These data indicate that α-SMA expression is dysregulated in IL-6KO mice. The expression of α-SMA induced by rmIL-6 in fibroblasts from IL-6KO mice appears to be transcriptionally modulated, dependent on JAK1 kinase, and possibly downregulated as a result of increased SOCS3 expression.

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INTRODUCTION

IL-6 is a pleiotropic cytokine that is involved in the growth and differentiation of numerous cell types including those of dermal and epidermal origin (Sehgal, 1990). In the skin, it is produced primarily by epidermal keratinocytes, whereas macrophages, Langerhans' cells, and fibroblasts in the dermis represent other sources of the cytokine (Paquet and Pierard, 1996). Increased levels of IL-6 have been associated with a number of skin pathologies, such as psoriasis (Grossman *et al.*, 1989), scleroderma (Koch *et al.*, 1993), and systemic lupus erythematosus (Fugger *et al.*, 1989). Overexpression of IL-6 in the skin of normal rats induces epidermal proliferation and inflammation (Sawamura *et al.*, 1998), whereas transgenic mice overexpressing IL-6 display little more than a thickened stratum corneum (Turksen *et al.*, 1992). In contrast to overexpressing transgenic animals, IL-6-deficient (IL-6KO) mice display significantly delayed cutaneous wound healing compared to wild-type control animals (Gallucci *et al.*, 2001b; Lin *et al.*, 2003). Wounds from IL-6KO mice displayed multiple defects including delayed re-epithelialization, greatly decreased granulation tissue, inhibited neovascularization, and delayed wound closure.

In the United States, over 6 million individuals develop chronic skin ulcers annually (Singer and Clark, 1999), and the restoration or augmentation of cutaneous wound healing has long been an elusive goal for health-care professionals. Following trauma to the skin, these diverse cell types of the dermis and epidermis must interact and cooperate in an orderly sequence of events that, when simplified includes coagulation, inflammation, formation of a provisional collagen matrix (granulation tissue), re-epithelialization, and remodeling (for a review see Bello and Phillips, 2000).

Wound closure is an important process in the proper healing of skin. The contraction of wound tissue is primarily accomplished by so-called myofibroblasts, which form from normal dermal fibroblasts (for a review see Tomasek *et al.*, 2002). During differentiation to the smooth muscle-like myofibroblast phenotype, dermal fibroblasts begin to express contractile proteins such as non-muscle myosin and α-smooth muscle actin (α-SMA), and orient themselves horizontally in the dermis

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Abbreviations: α-SMA, alpha-smooth muscle actin; IL-6KO, IL-6deficient; IL-6R, IL-6 receptor; rmIL-6, recombinant mouse IL-6; SOCS, suppressors of cytokine signaling; STAT, signal transducers and activators of transcription; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; Act-D, Actinomycin-D

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(Hinz *et al.*, 2001). In this position, the cells can contract in the granulation tissue to decrease the area of the wound.

Although it is known that wound closure is dependent on myofibroblast activity, the process by which dermal fibroblasts differentiate to this phenotype is not entirely understood. There are indications that certain cytokines and growth factors may be involved, such as platelet-derived growth factor (Martin, 1997), transforming growth factor (TGF)-β1 (Ronnov-Jessen and Petersen, 1993; Vaughan *et al.*, 2000), and inflammatory cytokines such as tumor necrosis factor-α (Mews *et al.*, 2002).

Herein, we describe the modulation of α-SMA expression in dermal fibroblasts from IL-6KO mice by the inflammatory cytokine, IL-6. We show not only that α-SMA is dysregulated in IL-6KO mice, but also that recombinant mouse IL-6 (rmIL-6) can modulate α-SMA in primary fibroblast cultures. Modulation *in vitro* is not merely upregulation of the protein, but rather a variable regulation of expression where low levels of IL-6 increase expression, and higher levels inhibit it. These data may have important implications in the development of treatments for chronic wounds, as well as for pathologies associated with myofibroblast disregulation such as Dupuytren's disease, or contractures from burn injury.

RESULTS

Modulation of wound α-smooth muscle actin in IL-6KO mice

As stated in the Introduction, IL-6KO mice display significantly decreased wound closure as compared to wild-type controls (Gallucci *et al.*, 2001b; Lin *et al.*, 2003). As the myofibroblast is primarily responsible for wound contraction, and α-SMA is a marker for myofibroblast differentiation, mRNA expression of α-SMA was measured in IL-6KO and wild-type control wounds. Time course analysis of wound α-SMA mRNA by real-time PCR analysis revealed that IL-6KO mice express significantly higher (~9-fold) levels of α-SMA up to 24 hours post wounding (Figure 1). However, 48 hours post-wounding α-SMA levels were not significantly different between groups, and by 72 hours IL-6KO mice expressed 2-fold less α-SMA than control mice. By 5 days α-SMA expression had return to levels equivalent to control. Immunohistology of wound tissue sections revealed that basal α-SMA expression in normal skin did not appear to be different between wild-type and IL-6KO mice (Figure 2a and b). Contrary to mRNA expression, 1 day post wounding no increase in fibroblast α-SMA protein was evident anywhere in the wound tissue including cellular infiltrate (Figure 2c and d). Indeed, little if any granulation tissue or α-SMA protein expression was evident in fibroblasts up to 96 hours post wounding (not shown). Five days after wounding, increased α-SMA protein staining was clearly evident in granulation tissue of C57 wild-type mice (Figure 2e). IL-6KO mice displayed less overall granulation tissue formation as previously reported (Gallucci *et al.*, 2001a), yet in the tissue that had formed very little α-SMA protein was evident (Figure 2f).

Modulation of α-smooth muscle actin mRNA expression in fibroblast culture

Epidermal keratinocytes as well as Langerhans' cells and dermal fibroblasts readily produce IL-6 (Grellner, 2002), and dermal fibroblasts from IL-6KO mice provide a useful model

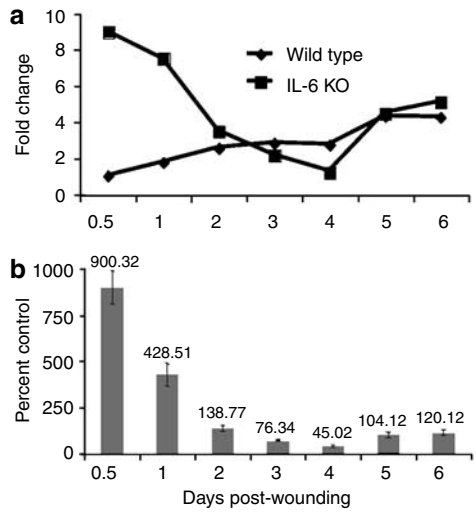

Figure 1. Time course of wound α-SMA mRNA in IL-6KO mice – real-time PCR analysis. IL-6KO or C57BL/6 mice were wounded, and mRNA was extracted from wound tissue as described. Expression of mRNA was determined via real-time PCR utilizing an ABI 7000 SDS system (Applied Biosystems), and accompanying software. Expression differences were determined relative to 18s rRNA utilizing the Comparative threshold cycle method. (**a**) Changes in expression over time, diamonds = wild-type control, squares = IL-6KO mice. (**b**) Percent IL-6KO expression of α-SMA relative to wild-type control, data are expressed as mean ± standard deviaton ($n = 5$ animals per data point).

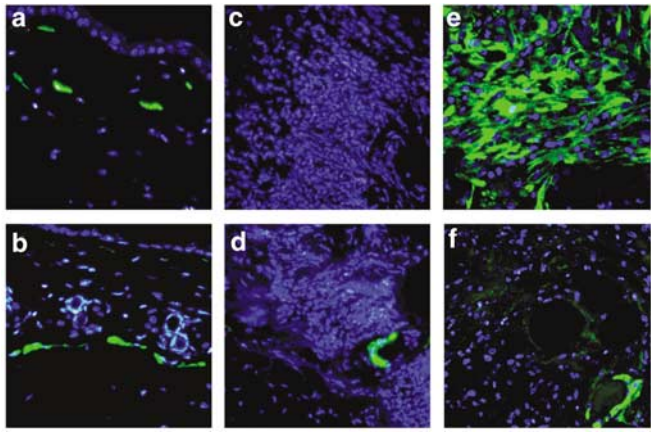

Figure 2. Expression of α-SMA in wound tissue. IL-6KO or C57BL/6 mice were wounded and wound tissue was collected at various time points after injury. Formalin-fixed, paraffin-embedded tissue sections of wound tissue were immunohistologically stained with anti α-SMA (green) and nuclear stain (dapi, blue). (**a** and **b**) Unwounded C57BL/6 and IL-6KO skin, respectively. (**c** and **d**) C57BL/6 and IL-6KO wound infiltrating cells 24 hours post injury. (**e** and **f**) C57BL/6 and IL-6KO wound granulation tissue 5 days post injury.

for the determination of the effects of IL-6 as intact IL-6R are expressed making them responsive to the cytokine, but no bioactive IL-6 is produced making antagonists unnecessary (Kopf *et al.*, 1994). To further assess the effect of rmIL-6 on α-SMA expression in fibroblasts, primary dermal fibroblasts from IL-6KO mice were cultured on tissue culture plates, and treated with various concentration of rmIL-6 for 2 hours as described. Total RNA was isolated and α-SMA expression was determined by real-time quantitative PCR. IL-6 appears to

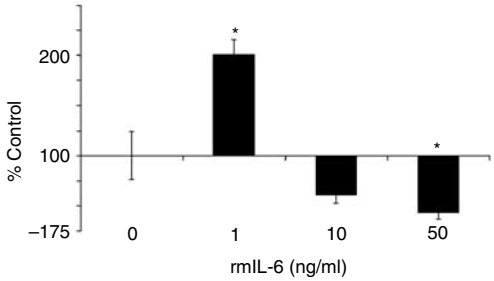

Figure 3. IL-6 modulates α-SMA mRNA expression in fibroblast culture. Primary dermal fibroblasts from IL-6KO mice were grown to 80% confluence on 100 mm tissue culture plates, and serum/growth factor starved for 4 hours. Semiconfluent cultures were treated with various concentrations of rmIL-6, and mRNA was isolated from treated fibroblasts after a 2-hour incubation. Expression of mRNA was determined via real-time PCR utilizing an ABI 7000 SDS system (Applied Biosystems), and accompanying software. Expression levels are represented relative to 18s rRNA utilizing the ddCt method. Data are expressed as mean ± standard error ($n = 5$ replicate experiments) percent saline control. (*significantly different from serum-free media control, $P < 0.05$).

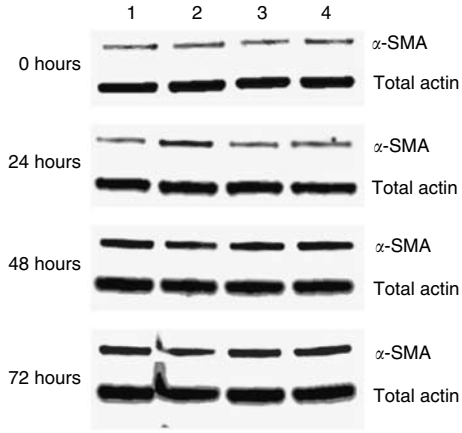

Figure 4. IL-6 induces α-SMA protein expression in fibroblast culture. Primary dermal fibroblasts from IL-6KO mice were grown to 80% confluence on 100 mm tissue culture plates. Growth media was replaced with low serum (0.1% fetal bovine serum) media and the semi-confluent cultures were treated with various concentration of rmIL-6 for 24 hours. Total cellular protein was prepared, and α-SMA expression was determined via Western blot. Lane 1: 0.0 ng/ml rmIL-6, lane 2: 1.0 ng/ml rmIL-6, lane 3: 10 ng/ml rmIL-6, lane 4: 50 ng/ml rmIL-6.

have variable effects on α-SMA mRNA expression in cultured IL-6KO fibroblasts (Figure 3). At 1 ng/ml, rmIL-6 significantly induced α-SMA mRNA (~2-fold), whereas concentrations of 10 and 50 ng/ml showed a progressive decrease of expression up to approximately 2-fold below control.

Modulation of α-smooth muscle actin protein expression in fibroblast culture

To determine whether protein levels of α-SMA were modulated similarly to mRNA, IL-6KO dermal fibroblasts were treated with various concentrations of rmIL-6 in low serum media, and total protein was collected after 0, 24, 48, and 72 hours incubation. Protein expression of α-SMA (relative to total actin) was determined by Western blot. Figure 4 shows a clear induction of α-SMA protein at 1 ng/ml rmIL-6 after 24 hours. Concentrations of >1 ng/ml did not significantly increase α-SMA protein compared to control. Incubations of >24 hours showed marked induction of α-SMA protein in fibroblast cultures regardless of treatment.

Transcriptional activation of α-SMA expression *in vitro*

To determine whether α-SMA mRNA increases were transcriptionally modulated, dermal fibroblast cultures were treated with rmIL-6 to induce α-SMA expression, and exposed to the transcriptional inhibitor Actinomycin-D (Act-D). One hour after addition of 1 ng/ml rmIL-6, Act-D (50 μg/ml) was added, and RNA samples were isolated at various time points after inhibitor treatment. The expression of α-SMA was upregulated approximately 3-fold at the time of transcriptional inhibition (Figure 5). However, α-SMA mRNA content decreased significantly 5 minutes post-Act-D addition, and remained not significantly different from control for the remainder of the experiment (40 minutes).

IL-6 treatment causes extracellular signal-regulated kinase 1/2 phosphorylation in dermal IL-6KO fibroblasts

The IL-6 receptor can activate both the JAK/signal transducers and activators of transcription (STAT), and extracellular

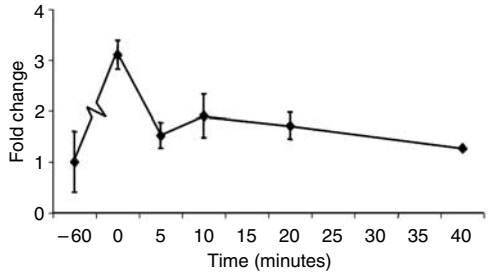

Figure 5. Act-D does not stabilize IL-6 induced α-smooth muscle actin mRNA. Primary dermal fibroblasts from IL-6KO mice were grown to 80% confluence on 100 mm tissue culture plates then incubated with 1 ng/ml rmIL-6 for 1 h. Act-D (5.0 μg/ml) was added to the cultures and RNA collected from cells at the indicated time points. Expression of α-SMA was determined by real-time PCR utilizing an ABI 7000 SDS system (Applied Biosystems), and accompanying software. Expression levels are expressed relative to 18s rRNA utilizing the ddCt method. Graphic representation of the image analysis is shown.

signal-regulated kinase (ERK) kinase signal transduction pathways. To determine which transduction pathways were induced in dermal IL-6KO fibroblasts, protein samples were taken from 1 to 20 minutes following treatment with 1 ng/ml rmIL-6. ERK1/2 phosphorylation was apparent after 1 minute, while reaching a maximal level 5 minutes post-treatment, whereas increased phospho-STAT3 when compared to total STAT3 protein was not increased at any time point (Figure 5).

JAK1 and ERK kinase inhibition decreases α-SMA expression in cultured IL-6KO fibroblasts

Because ERK1/2 was rapidly activated following IL-6 treatment, and phosphorylation of this kinase is mediated by JAKs, specific signal transduction inhibitors were utilized to determine which kinases were necessary for the induction of α-SMA expression. Dermal fibroblasts from IL-6KO

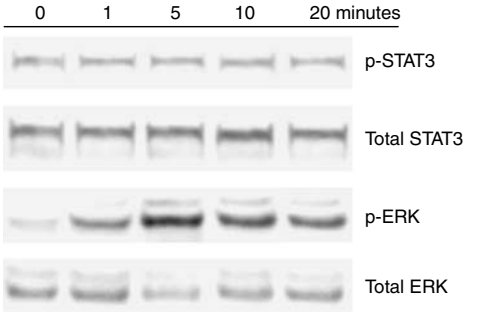

Figure 6. IL-6 treatment activates ERK1/2 but not STAT3 phosphorylation in dermal fibroblasts. Primary dermal fibroblasts from IL-6KO mice were grown to 80% confluence on 100 mm tissue culture plates, and treatments were applied as indicated. Total cellular protein was isolated from the treated cultures at the indicated time points. Western blot analysis was performed utilizing 25 μg of total protein, and bands were detected with anti-phosphoprotein antibodies (Cell Signaling Tech., Beverly, MA). Blots were imaged using a Molecular Imager FX Pro Plus (BioRad, Hercules, CA). Photographs are representative of three identical experiments.

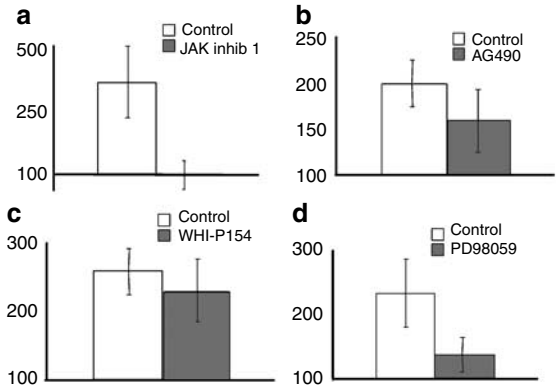

Figure 7. Inhibition of JAK and ERK modulation of α-SMA mRNA expression. Primary dermal fibroblasts from IL-6KO mice were grown to 80% confluence on 100 mm tissue culture plates. Cultures were treated with specific kinase inhibitors for (**a**) pan-JAK (JAK inhibitor I) (**b**) JAK2 (AG490), (**c**) JAK3 (WHI-P154), and (**d**) ERK (PD98059) for 30 minutes. Semiconfluent cultures were treated with 1 ng/ml rmIL-6, and mRNA was isolated from treated fibroblasts after 2 hours. Expression of mRNA was determined via real-time PCR utilizing an ABI 7000 SDS system (Applied Biosystems), and accompanying software. Expression differences were determined relative to 18s rRNA utilizing the ddCt method.

mice were treated with pan-JAK (JAK inhibitor I, 10 μM), JAK2 (AG490, 40 μM), JAK3 (WHI-P154, 10 μM), or ERK1/2 (PD98059, 10 μM) inhibitors for 30 minutes before addition of 1 ng/ml rmIL-6. Two hours post-rmIL-6 treatment α-SMA mRNA expression was assessed. JAK inhibitor I and PD98059 significantly decreased α-SMA expression (Figure 6a), whereas inhibitors of JAK2 and 3 showed slight but not significant inhibition (Figure 6b–d).

Suppressors of cytokine signaling 3 is induced in fibroblast cultures following IL-6 treatment

IL-6R signaling initiated JAK/STAT signaling is regulated by a feedback mechanism that involves suppressor of cytokine signaling 3 (SOCS3) (Heinrich *et al.*, 2003). Figures 2 and 3 show that concentrations of rmIL-6 $\geqslant$10 ng/ml do not significantly induce α-SMA mRNA or protein expression (respectively). Because it appears as though α-SMA expression is dependent on JAK activity, rmIL-6-induced SOCS3 expression in IL-6KO fibroblasts was examined. Dermal fibroblasts from IL-6KO mice showed a dose–response increase in SOCS3 in response to rmIL-6 treatment, ranging from a 2-fold increase at 1 ng/ml to a nearly 7-fold increase at 50 ng/ml (Figure 7).

DISCUSSION

IL-6KO mice display greatly impaired wound healing as evidenced by delayed wound closure, incomplete re-epithelialization, decreased inflammation, granulation tissue formation, and neovascularization (Gallucci *et al.*, 2000; Lin *et al.*, 2003). This impairment was noted for as long as 15 days, nearly triple the normal healing time. IL-6KO mice express normal levels of functional IL-6 receptor (Kopf *et al.*, 1994), and wound healing could be reconstituted by treating IL-6KO mice with rmIL-6 (Gallucci *et al.*, 2001a; Lin *et al.*, 2003) or an IL-6 expression plasmid (Gallucci *et al.*, 2001a). Whereas wound closure is significantly inhibited in IL-6KO mice, and it has been shown that the expression of α-SMA, a differentiation marker of the myofibroblast, is induced in pancreatic fibroblast cultures by IL-6 treatment (Mews *et al.*, 2002), it is unclear what role IL-6 plays in the regulation of expression of this contractile protein in the skin during healing. Herein, we show that IL-6KO mice display dysregulated α-SMA expression during wounding, and that rmIL-6 can both induce and inhibit fibroblast α-SMA expression *in vitro*.

IL-6 is expressed in wounded skin in two distinct phases. IL-6 is initially upregulated quickly after skin injury, with robust protein levels peaking approximately 12 hours post injury, but drops to near basal levels by 24 hours (Mateo *et al.*, 1994). The second phase of IL-6 expression in the wound, begins after approximately 48 hours, where cytokine levels gradually reach steady state, albeit at a lower tissue concentration than the initial pulse, from 3–7 days post injury (Mateo *et al.*, 1994).

Despite the fact that there is evidence in the literature that IL-6 can modulate the expression of α-SMA, the role it plays in skin appears to be complicated and sometimes conflicting. Cell culture data presented herein seem to support the notion that IL-6 upregulates α-SMA mRNA expression quickly, corresponding with the rapid activation of IL-6R signal transduction. However, in normal wounds, α-SMA expression is not apparent in granulation tissue until approximately 5 days post injury (Tomasek *et al.*, 2002). Furthermore, the expression of α-SMA mRNA following wounding of IL-6KO mice is initially ($\sim$24 hours) greatly increased compared to control (Figure 1), indicating that IL-6 might play an inhibitory role in α-SMA expression, and seemingly contradicting our *in vitro* data. This is further complicated by the fact that α-SMA mRNA levels do not seem to follow protein levels in fresh wounds in IL-6KO mice (Figure 2).

The observed discrepancies between *in vitro* and *in vivo* results, as well as mRNA and protein expression are not

entirely unexpected as the regulation of α-SMA expression has been shown to be controlled at multiple levels. For instance, the expression of α-SMA mRNA is readily detected in bovine brain microvessels, yet no protein is detected in capillary endothelial cells or pericytes by immunohistology (Boado and Pardridge, 1994). In the developing heart tissue of chicken embryos, α-SMA mRNA is expressed much earlier than protein (Colas *et al.*, 2000). In fibroblast cultures, the DNA-binding proteins Purα, Purβ, as well as MSY1 (Kelm *et al.*, 1999), and its mouse homologue YB-1 (Zhang *et al.*, 2005) are known to activate transcription of α-SMA, yet also shuttle into the cytosol, bind to its mRNA, and inhibit translation of the sequence. Thus, unique checkpoints seem to exist that regulate the expression of α-SMA at both transcriptional and post-transcriptional levels. One purpose for multilevel control of this gene may be to inhibit its inappropriate expression. For instance, increased α-SMA expression inhibits fibroblast motility (Ronnov-Jessen and Petersen, 1996), and myofibroblasts themselves inhibit re-epithelialization (Moulin *et al.*, 2000). Thus, it would seem necessary for both fibroblast migration and re-epithelialization to proceed before myofibroblast differentiation for proper healing to occur. Therefore in the early wound, high levels of expression of IL-6 might activate one of these ''checkpoints'', and inhibit early inappropriate α-SMA expression at the transcriptional level. Indeed, we find that higher concentrations (>1 ng/ml) inhibit α-SMA expression *in vitro* (Figure 3). However, it is not known whether the aforementioned regulatory mechanisms are associated with IL-6R signaling, and it seems clear that IL-6 is just one of multiple signals that affect α-SMA expression in fibroblasts, as a translational block seems to function quite well in IL-6KO mice in the early phases of healing (Figure 2).

The second phase of IL-6 expression during wound healing (>72 hours) appears to coincide with myofibroblast formation and wound closure (see Tomasek *et al.*, 2002 for review). At this point, IL-6 expression is lower than the initial pulse, yet more persistent (Mateo *et al.*, 1994). Although we do not show the direct effects of IL-6 on fibroblasts *in vivo*, it was found that IL-6KO mice express markedly decreased α-SMA protein levels in granulation tissue after 5 days (Figure 2), and low concentrations of IL-6 induce the expression of α-SMA in IL-6KO fibroblast culture (Figures 3 and 4). Thus, we postulate that perhaps by this stage of healing, IL-6 might aid in myofibroblast function and wound closure by promoting fibroblast differentiation as defined by α-SMA expression. Interestingly, Figure 4 indicates that fibroblast cultures display increased α-SMA protein expression that corresponded with age of culture, which was irrespective of IL-6 treatment beyond 24 hours. Indeed, it is known that when cultured on tissue culture plastic, fibroblasts will spontaneously differentiate toward a myofibroblastic phenotype (Tomasek *et al.*, 2002), as a result of tractional forces encountered and this is especially apparent in fibroblasts of mouse origin. Thus, it seems that IL-6 is one of many mediators that can modulate α-SMA expression, and may augment or work in concert with other factors to induce differentiation of dermal fibroblasts.

The fact that the modulation of α-SMA expression by IL-6 appears to be concentration dependent, it is not surprising as IL-6 is well known to be a very (arguably the most) pleiotropic cytokine, displaying a multitude of activities in various tissues (Sehgal, 1990). The reasons for this are largely unknown. A clue to this paradox may lie in the regulation of IL-6R signal transduction. The IL-6 receptor is composed of two basic subunits, the function and signal transduction of which has been extensively reviewed elsewhere (Heinrich *et al.*, 2003). IL-6 can initiate transduction through the IL-6Rβ (gp130) subunit leading to the rapid activation of numerous signaling paths including that of JAK/STAT3, and ERK/mitogen-activated protein kinase (MAPK). Three different JAK kinases (JAKs 1, 2, and 3) can interact with the IL-6R, to activate various STATs, and the MAPK pathway can be activated indirectly through recruitment of SH2 containing protein tyrosine phosphatase 2. The MAPK pathway can activate several transcription factors including AP-1, CEBPβ, and NF-κB, as well as Elk-1 which can affect smooth muscle gene expression through the modulation of serum response factor activity (Meyyappan *et al.*, 1996; Sobue *et al.*, 1999; Wang *et al.*, 2004).

The present study demonstrated that the induction of α-SMA mRNA in IL-6-treated IL-6KO fibroblasts seems to be at least partially transcriptionally regulated as the transcriptional inhibitor Act-D treatment greatly decreases this genes mRNA levels (Figure 5). To further investigate the relationship between IL-6R signaling events and α-SMA gene transcription, the JAK/STAT and ERK signaling pathways were assessed in rmIL-6-treated fibroblasts from IL-6KO mice by Western blot. It was previously reported that 25 ng/ml rmIL-6 induced rapid STAT3 activation in cultured IL-6KO fibroblasts, whereas ERK1/2 was not modulated (Gallucci *et al.*, 2004). Interestingly, in the present study it was found that 1 ng/ml rmIL-6 induced only ERK1/2 phosphorylation in cultured IL-6KO fibroblasts. ERK1/2 required approximately 5 minutes to reach maximum phosphorylation, whereas STAT3 phosphorylation levels remained constant throughout the experiment (Figure 6). Whereas this indicated that primarily the MAPK pathway was induced, the IL-6R modulates this pathway through JAK-mediated phosphorylation of SH2 containing protein tyrosine phosphatase 2 as stated earlier. Thus, it was not clear which JAK enzymes contributed to the induction of α-SMA expression. To investigate this further, cultured IL-6KO fibroblasts treated with 1 ng/ml rmIL-6 were exposed to specific inhibitors for each of the three JAK kinases, and MAPK. Of the four treatments only the inhibitors JAK-inhibitor I (pan JAK inhibitor) and PD98059 (ERK) produced a significant decrease in α-SMA mRNA expression (Figure 7a and d). Inhibitors of JAK2 and 3 did not significantly decrease α-SMA mRNA expression, indicating that JAK1 is initially responsible for the induction of this gene. This is not surprising as JAK1 is activated preferentially by the IL-6R and responsible for the majority of its signaling activity (Heinrich *et al.*, 2003). It should be noted that inhibition of JAK2, and 3 did slightly decrease α-SMA expression (Figure 7b and c), and thus may have minor roles in the modulation of this gene. However, currently there are no studies that

directly implicate the JAK/STAT or MAPK pathways in the induction of α-SMA gene expression.

Similar to induction, the inhibition of α-SMA mRNA expression in IL-6KO fibroblasts by IL-6 may occur through several routes, as there are several mechanisms by which IL-6 signaling can be terminated. There is significant cross-talk between JAK/STAT and other signaling pathways. For instance, SMA and MAD related protein 3 transactivation is necessary for transforming growth factor-β-induced α-SMA expression (Hu *et al.*, 2003), and transforming growth factor-βR/SMA and MAD related protein 3 signaling is inhibited by IFN-γ activation of JAK1, through the induction of the inhibitory transcription factor SMA and MAD related protein 7 (Ulloa *et al.*, 1999). Similarly, SMA and MAD related protein 3 and 4 can inhibit IL-6 signaling (Zauberman *et al.*, 2001). Additionally, it is well known that IL-6 signaling is regulated by a multicomponent negative feedback loop (for a review see Heinrich *et al.*, 2003). For instance, the family of IL-6R signaling inhibitors, known as suppressors of cytokine signaling (SOCS), inhibit JAK activity, and IL-6R activation specifically upregulates the expression of SOCS1–3. Indeed, SOCS3 is induced in rmIL-6-treated IL-6KO fibroblasts in a dose–response manner (Figure 8). As JAK activation is necessary for IL-6R-mediated MAPK signal propagation, it could be that SOCS proteins are responsible for the down-regulation of α-SMA in cultured IL-6KO fibroblasts at higher concentrations of IL-6.

When considered with previous articles, the present study supports the hypothesis that IL-6 assists skin wound healing at multiple levels most likely by virtue of its highly pleiotropic nature, and variable expression during skin wound healing. It may be that robust early wound IL-6 expression promotes early events such as keratinocyte migration through the modulation or maintenance of fibroblast phenotype. Whereas, later lesser upregulation of the cytokine, in combination with other factors, could assist in wound closure by inducing the myofibroblast phenotype. Although it does appear that IL-6 plays a complex role in the modulation of skin α-SMA expression *in vivo* and *in vitro*, the exact mechanism by which this occurs remains unknown. Clearly, further research will be necessary to determine the exact the role of IL-6 in wound closure and healing.

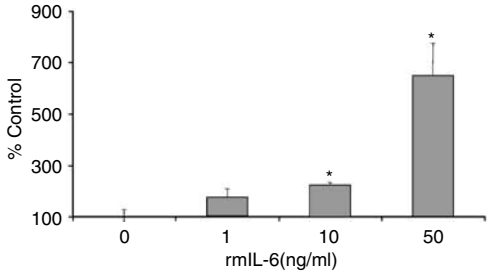

Figure 8. IL-6 induces SOCS3 mRNA expression in fibroblast culture. Primary dermal fibroblasts from IL-6KO mice were grown to 80% confluence on 100 mm tissue culture plates, and serum/growth factor starved for 4 hours. Semiconfluent cultures were treated with various concentration of rmIL-6, and mRNA was isolated from treated fibroblasts after a 2-hour incubation. Expression of mRNA was determined via real-time PCR utilizing an ABI 7000 SDS system (Applied Biosystems), and accompanying software. Expression levels are expressed relative to 18s rRNA utilizing the ddCt method. Data are expressed as mean ± standard deviaton ($n = 5$) percent saline control (*significantly different from serum free media control, $P < 0.05$).

MATERIALS AND METHODS

Reagents

rmIL-6 was purchased from Invitrogen (Carlsbad, CA). The signal transduction inhibitors, AG490 (JAK2), JAK inhibitor I (pan-JAK), WHI-P154 (JAK3), and PD98059 (ERK1/2) were purchased from EMD Biosciences (San Diego, CA).

In vivo wounding

Experimental animals were treated in accordance with the criteria outlined in the PHS Policy on Humane Care and Use of Laboratory Animals, and the ''Guide for the Care and Use of Laboratory Animals'' (NIH publication 86–23), in facilities accredited by Association for Assessment and Accrediation of Laboratory Animal Care. IL-6-deficient (C57BL/6J-*IL6*^{tmKopf}) or wild-type (C57BL/6J) mice were wounded as previously described (Gallucci *et al.*, 2001a). Briefly, male wild-type mice or IL6KO mice (Jackson Laboratories, Bar Harbor, ME) weighing 22–28 g and approximately 8–12 weeks old were housed in polycarbonate cages containing hardwood chip bedding at room temperature (21 ± 2°C) on a 12-hour light/dark cycle. Mice were anesthetized by intraperitoneal injection with 80 mg/kg pentobarbital, and the left flank was clipped and swabbed with Betadine (Purdue Fredrick Co., Norwalk, CT) and 70% ethanol three times before wounding. Four millimeter punch biopsies were performed on the shaved area. After various healing periods, wound tissue was collected (2–3 mm border was excised around the wound) and preserved by flash freezing in liquid nitrogen.

Dermal fibroblast isolation and culture

The dermis was collected and separated from the epidermis as described previously (Gallucci *et al.*, 2004). Briefly, newborn IL-6KO mouse pups (0–48 hours old) were killed by decapitation, rinsed in 70% ethanol, and the skin was removed. Excised mouse skin was placed in culture dishes dermis side down, in Hank's balanced salt solution + 3.6% dispase (Sigma, St. Louis, MO) and incubated at 4°C overnight. The dermis was separated from the epidermis, placed in Hank's balanced salt solution + 0.05% Type 1 collagenase and incubated at 37°C for 30 minutes with constant agitation. The cell suspension was filtered through sterile gauze into a 50 ml conical tube. An equal volume of DMEM + 5% fetal bovine serum containing 60 IU/ml penicillin, 100 IU/ml streptomycin, and 4 mM glutamine (DMEM + 5%) was added and centrifuged at 200 × *g* for 10 minutes. Fibroblasts were resuspended in DMEM + 5%, plated at $8 \times 10^3/cm^2$ in 150 mm culture dishes, and incubated at 37°C and 5% CO_2 and 95% room air. The primary IL-6KO fibroblasts were passaged once and utilized for subsequent experiments. Dermal cells were cultured in DMEM which does not support keratinocyte growth, and the cells were passaged once to minimize contamination from these cells, but not passaged further as cultures begin to take on a proto-myofibroblast (Tomasek *et al.*, 2002) appearance and greatly increase α-SMA expression (unpublished observations). Cells were phenotyped by gross morphology, and possessed the classical long spindle shape of fibroblasts.

Real-time quantitative PCR

Primary dermal fibroblasts from IL-6KO mice were grown to 80% confluence on 100 mm tissue culture plates, and serum/growth factor starved for 4 hours. Semiconfluent cultures were treated with various concentrations of rmIL-6, and mRNA was isolated from treated IL-6KO fibroblasts after a 2-hours incubation. The IL-6R signal transduction pathway is very rapidly activated following IL-6 binding, resulting in mRNA induction within seconds to minutes (Heinrich *et al.*, 2003). Shorter (1–2 hours) time points, as were chosen for the present study, allow for sufficient accumulation of mRNA, yet indicate a more direct modulation as a result of IL-6 treatment as the influence of *de novo* protein synthesis is minimal. Total RNA from mouse skin or dermal IL-6KO fibroblasts was prepared and cDNA synthesized as described previously (Gallucci *et al.*, 2004). Primers and probes for mouse α-SMA, SOCS3, and 18s rRNA were acquired from Applied Biosystems (Assays-on-demand, Foster City, CA). Quantitative PCR was performed on an ABI PRISM 7000 SDS according to the manufacturer's instructions. Quantitative values of genes of interest are normalized based on 18s rRNA content.

Immunohistology

Anti-α-SMA and Alexa 488 anti-mouse antibodies were obtained from Sigma (St Louis, MO). Formalin-fixed paraffin-embedded sections were dewaxed, permeabilized in 0.1% NP40, and stained for α-SMA essentially as previously described (Tomasek *et al.*, 2005). Slides were washed, coverslipped, and staining was visualized by fluorescent microscopy.

Western blot analysis

IL-6KO fibroblasts were prepared as described above, and cultured to 90% confluency in 150 cm^2 culture plates. Before treatment, fibroblast cultures were washed twice with phosphate-buffered saline, and culture media was replaced with low serum (0.1% fetal bovine serum) DMEM containing, 60 IU/ml penicillin, 100 IU/ml streptomycin, and 4 mM glutamine. Low serum medium was necessary to maintain viability of primary fibroblasts overnight. Following a 4-h incubation in low serum medium, treatments were applied to the cultures in fresh low serum DMEM. At indicated time points, culture plates were rinsed once with ice-cold phosphate-buffered saline, and total cell protein was collected in 150 μl of 2 × Laemmli denaturing sample buffer (Sigma #S3401). Approximately 25 μg of total protein was electrophoresed on 10% SDS-PAGE (10 cm × 10 cm) minigels (Gradipore, Frenchs Forest, AUS) at 90 V for 45 minutes, and electrophoretically transferred onto nitrocellulose sheets (Pierce, Rockford, IL) at 25 V for 2.5 hours. Following transfer, the blots were blocked in Tris-buffered saline containing 1% cold-water fish gelatin (Sigma, St Louis, MO), and 50 mM NaF for 1 hour at room temperature. Monoclonal anti-α-SMA, rabbit anti-actin (Sigma), or anti-p-STAT3/total STAT3, p-ERK1/2/total ERK1/2 (Cell Signaling, Beverly, MA) were applied to the blots at a 1:1000 dilution in Tris-buffered saline containing 1% fish gelatin, 0.1% Tween 20, and 50 mM NaF overnight at 4°C. The blots were washed 3 × in Tris-buffered saline containing 0.1% Tween 20, and exposed to a fluorescently labeled secondary anti-species polyclonal antibody(s) (Rockland, Gilbertsville, PA) at a 1:30,000 dilution in Tris-buffered saline containing 1% fish gelatin and 0.1% Tween 20 for 1 hour. Following three washes in Tris-buffered saline + 0.1% Tween, the blots were imaged via the Molecular Imager ProPlus imaging system (BioRad, Hercules, CA).

Statistical analysis

All experiments were replicated and representative findings are shown. Statistical significance was determined by one-way analysis of variance. When the F-value was significant, the means were compared using Fisher's *post hoc* analysis. In all statistical comparisons, a P-value of <0.05 was used to indicate a significant difference.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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